A PHOSPHATASE TEST FOR DETERMINING HEAT TREATMENT OF ALFALFA MEAL

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Alfalfa meal of highest nutritional value for use in feeds can be prepared only from hay that has been heat-dehydrated promptly after cutting. Alfalfa is dehydrated artificially in a current of hot air to preserve its quality and nutritional value and especially to reduce the loss of carotene, much of which is destroyed by exposure to sun and weather in field curing. Carotene, precursor of vitamin A, is a very valuable nutritional constituent of livestock feeds. Dehydrated alfalfa meal, used as a component of feeds for poultry and animals, is an important and practical source of carotene.

Shepherd *et al. (8)* pointed out that dehydration, entailing a shorter period of exposure in the field and a shorter time from cutting to storage than other harvesting methods, results in smaller losses of leaves, digestible nutrients, green color, and carotene. Certain investigations including analyses *(2, 3, 4, 8)* have shown that artificially dried and ground alfalfa may contain between six and 21 times as much carotene as field-cured alfalfa. Moreover, the stability of carotene in alfalfa is increased by heat treatment *(5, 9)*, and this increased stability is attributed largely to heat inactivation of the carotene-destroying enzyme system *(9)*, referred to also as carotene oxidase *(5)*.

Inferior and less expensive meal can be prepared at any time of the year by grinding field-cured hay. It is easily possible for feed manufacturers to adulterate the heat-dehydrated product by adding field-cured, ground hay to the meal. A sensitive, reliable test is needed, therefore, for detecting such adulteration.

Experiments were conducted to develop a phosphatase test for this purpose. The resulting test is similar to tests employed for detecting underpasteurization of dairy products. Like milk, alfalfa contains an alkaline phosphatase enzyme that is inactivated by heat. The temperatures employed in artificial dehydration of alfalfa—commonly 250° to 350° F. at the outlet—are far in excess of those employed in pasteurization and therefore are sufficient to inactivate the enzyme completely. As the enzyme remains active in field-cured hay, its presence in what is offered as heat-dehydrated meal indicates adulteration with field-cured hay or other nondehydrated material, or too low a temperature of dehydration.

Principle. Essentially, the method is based on the fact that alfalfa contains an enzyme, phosphatase, which is inactivated by heat. A sample of alfalfa is treated with disodium phenyl phosphate in a buffer at the proper pH. If the enzyme is present, phenol is liberated. Interfering proteins are removed by precipitation with trichloroacetic acid and filtration. The phenol-containing solution is then made alkaline and treated with "BQC," forming blue indophenol, whose concentration is a measure of the phosphatase present.

Reagents.

1. *Buffers:*

a. *Buffer for washing meal.* Dissolve 3.18 g. of anhydrous sodium carbonate (Na_2CO_3) and 5.88 g. of anhydrous sodium bicarbonate ($NaHCO_3$) in distilled water and dilute to 1 l. (pH 9.6).

b. *Incubation buffer.* Dissolve 8.0 g. of sodium carbonate and 4.0 g. of sodium bicarbonate in distilled water and dilute to 1 l. (pH 10.1).

c. *Color development buffer.* Dissolve 17.5 g. of sodium metaborate ($NaBO_2$) and 10.0 g. of sodium chloride (NaCl) in water and dilute to 1 l. (pH 10.2).

2. *Buffer substrate:*

Add 0.1 g. of phenol-free, crystalline disodium phenyl phosphate[1] (substrate) to 100 ml. of incubation buffer. If substrate contains free phenol, first extract phenol from it as described earlier *(1, 6)*.

3. *Precipitant:*

Dissolve 42.0 g. of trichloroacetic acid (CCl_3COOH) and 0.05 g. of copper sulfate ($CuSO_4 \cdot 5H_2O$) in water and dilute to 100 ml.

4. *BQC (2,6-dibromoquinonechloroimide):*

Dissolve 50 mg. of BQC[1] powder in 10 ml. of absolute ethyl or methyl alcohol and transfer to a brown dropper bottle. This reagent remains stable for at least a month if kept in the ice tray of a refrigerator. Do not use it after it begins to turn brown.

Preparation of test samples. Before testing, the samples must first be washed free of chlorophyll and other colored, interfering substances. The following preparation and washing procedure is recommended. If hay is to be tested, grind it thoroughly. A Wiley mill is suitable for grinding. Weigh 1.5 g. of sample (meal or ground hay) and transfer it to a 150-ml. beaker. Add 50 ml. of washing buffer (resulting pH about 9.3), stir well, and allow to soak 15 minutes with occasional stirring. Filter through a Büchner funnel with suction, using Reeve-Angel No. 711 filter paper or paper of equivalent characteristics. Wash five times with 50 ml. of water each time. Dry overnight at room temperature. Adequate dryness is indicated by meal not sticking to spatula when stirred.

[1] Obtainable relatively pure, from Applied Research Institute, 2 East 23rd St., New York 10, N. Y. Mention of this firm implies no especial endorsement by the Department of the quality of its product; nor is it recommended in preference to other firms not mentioned that may produce the same product.

Preparation of controls. The control samples must be heated to destroy phosphatase. Weigh 5 g. of unwashed meal, obtained from the same sample that is to be tested, into a 250-ml. beaker. Add 100 ml. of water and boil almost to dryness. Place in oven at 212° F. and leave overnight. Then weigh 1.5 g. and wash as described above.

Conducting the test. Weigh 0.25 g. of washed meal, prepared as described under *Preparation of test samples,* into a test tube 16 or 18 × 150 mm. Weigh 0.25 g. of heated, washed meal, prepared as described under *Preparation of controls,* into another test tube. From this point, treat tests and controls alike. Add 10.0 ml. of buffer substrate to each. Mix thoroughly and incubate in water bath at 99°-100° F. (37°-38° C.) for 60 minutes. Add 1.0 ml. of precipitant, slowly, and shake carefully. Cover the tube lightly and invert several times. Permit gas to escape occasionally to prevent excessive pressure and spattering. Filter contents through 9-cm. No. 42 Whatman or filter paper of equivalent characteristics. Transfer 5.0 ml. of filtrate to another tube (preferably graduated at 5.0 and 10.0 ml.) and add 5.0 ml. of color development buffer (pH of mixture 9.3-9.4). Add 2 drops of BQC solution and mix thoroughly. Appearance of blue color indicates the presence of material that has not been dehydrated with heat. Allow color to develop for 30 minutes at room temperature or 15 minutes at 99°-100° F. Evaluate intensity of blue color preferably photometrically, employing a 610-mμ filter, or visually by comparison with standards prepared with known quantities of phenol. Subtract the value of the control from that of the test. Details of methods for preparing standards and measuring color intensities are available in descriptions of the Sanders-Sager *(1, 6, 7)* phosphatase test.

Calculation of results: γ (micrograms or units) of phenol $\times \frac{11}{5} = \gamma$ of phenol per 0.25 g. of sample.

EXPERIMENTAL PROCEDURE

Experiments were conducted to determine the range of pH at which the phosphatase exhibited its activity. A series of phosphatase tests was made by using the foregoing procedure, except that just prior to incubation, varying increments of hydrochloric acid or sodium hydroxide were added to yield incubation pH values over a range of 4.6 to 10.8. The results in Figure 1 (optimal pH curve) show that alfalfa contains both acid and alkaline phosphatase, the pH optima being approximately 5.0 and 10.0, respectively, in test samples incubated for 1 hour. The phosphatase activity in unheated meal was generally about ten times as great at pH 5 as at pH 10. However, the acid enzyme was found to be more resistant to heat, only about 90% being inactivated by heating at the usual dehydration temperatures. The alkaline phosphatase was completely destroyed by heat dehydration, and therefore a test for alkaline phosphatase was employed.

Tests were conducted to determine phosphatase values of samples containing different proportions of field-cured hay meal in heat-dehydrated meal, to indicate results of different proportions of adulteration. Figure 2 shows the average of

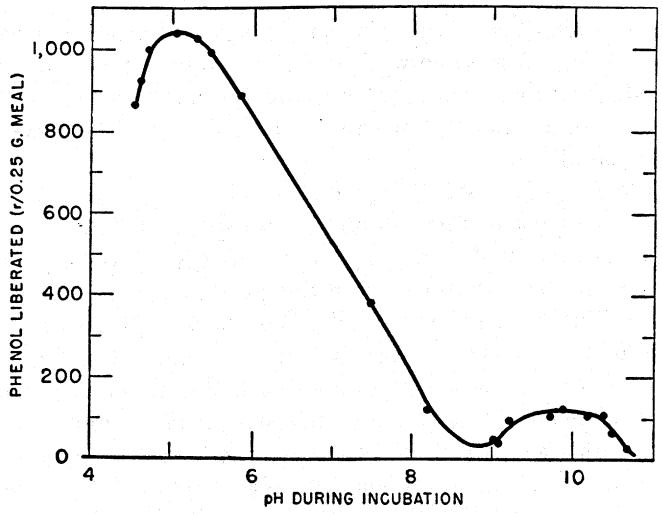

FIG. 1. Phenol liberated at different pH values in the phosphatase test on alfalfa meal prepared from field-cured (nonheat-dehydrated) hay, 1-hour incubation.

four phosphatase tests with each of eight different percentages of field-cured meal in heat-dehydrated meal. The blue color yielded by as little as 1 γ of phenol in the test, or approximately 2 γ of phenol per 0.25 g. of meal used, can be seen visually and readily detected. The data show the test to be sufficiently sensitive to detect as little as 1% adulteration with field-cured meal (phosphatase positive) added to properly heat-dehydrated meal (phosphatase negative). The test was only roughly quantitative with respect to the proportions between quantities of phenol liberated and percentages of adulteration, and a strictly linear (straight-line) relationship was not obtained (Figure 2).

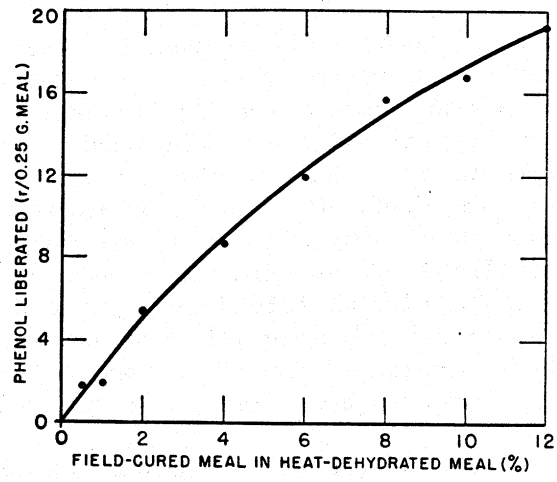

FIG. 2. Results of phosphatase tests on samples containing various percentages of field-cured alfalfa meal in heat-dehydrated alfalfa meal.

A phosphatase test for dairy products was published earlier by Sanders and Sager *(7)* and later was adopted as an official method *(1)* for determining whether or not milk and other dairy products have been adequately pasteurized. The methodology is very similar to that of the test described above, the principal differences being in the buffers and the precipitant.

In developing a practical and effective test for alfalfa meal, it was of interest to compare results obtained by the test described herein with those obtained by the Sanders-Sager (S-S) test. All the details of the S-S procedure applied to alfalfa meal were the same as the S-S procedure applied to milk, except as follows: A 0.25-g. sample of meal was used, the test samples were not heated after incubation, the protein precipitant contained 5.0 g. of zinc sulfate and 0.5 g. of copper sulfate diluted to 100 ml. in water, and the color development buffer contained 8.0 g. of sodium metaborate and 20.0 g. of sodium chloride per liter.

Table 1 shows comparative data obtained with the S-S procedure and with the Sanders-Hupfer (S-H) procedure as described above. The S-S test yielded lower control values (less off color) than did the S-H test. However, the S-H test was somewhat more sensitive in detecting the presence of field-cured alfalfa meal, and accordingly it is recommended in preference to the S-S test for this purpose.

DISCUSSION

Chlorophyll and other compounds in alfalfa interfere in the test and must be removed before testing. Dilute alkaline solutions are effective for this purpose. A sodium carbonate-bicarbonate washing buffer, described above under *Reagents,* was selected because it effectively removes interfering chromophoric compounds and its pH is not so high as to cause appreciable inactivation of the enzyme.

The alkaline phosphatase in alfalfa is considerably more resistant to heat inactivation than the phosphatase in milk. Its heat resistance varies somewhat in different varieties and samples of alfalfa. With some varieties, heating a 0.25-g. sample in 3.0 ml. of incubation buffer to 203° F. (95° C.) is sufficient for complete inactivation. The alkaline enzyme in all varieties and samples tested was completely inactivated by prolonged heating in water at 212° F., described above under *Preparation of controls.*

This method may not detect the presence of field-cured hay that has been subsequently heated.

SUMMARY

Alfalfa meal of the best quality for use in feeds must be prepared by artificial heat dehydration to minimize destruction of carotene or pro-vitamin A and to reduce deterioration of other nutritive qualities. A modified phosphatase test was developed to fill the need for a reliable, sensitive method for detecting adulteration of heat-dehydrated alfalfa with nutritionally inferior and less expensive field-cured alfalfa and other nonheat-treated materials.

Alfalfa hay contains both acid phosphatase and alkaline phosphatase. Only the alkaline enzyme is completely inactivated by usual temperatures employed

TABLE 1

Results of phosphatase tests on alfalfa hay meal

Sample No.	Sanders-Sager procedure[a]				Sanders-Hupfer procedure[b]			
	Photometric reading[c]			Phenol equivalent	Photometric reading			Phenol equivalent
	Sample	Control (heated)	Difference		Sample	Control (heated)	Difference	
				(γ/0.25 g.)				*(γ/0.25 g.)*
				Heat-dehydrated meal				
H-D 1	72	70	2	0[d]	72	67	5	0
	65	64	1	0	73	68	5	0
2	88	83	5	0	77	81	−4	0
3	78	78	0	0	85	90	−5	0
Av.	76	74	2	0	77	77	0	0
				Field-cured hay meal				
F-C 1	335	70	265	49	552	77	475	87
	295	70	225	41	470	80	390	71
2	565	65	500	92	578	66	512	94
	491	71	420	77	631	66	565	104
3	1780	80	1700	312	1483	98	1385	254
4	236	81	155	28	319	99	220	40
5	860	70	790	145	761	76	685	126
6	944	99	845	155	838	103	735	135
7	—	—	—	—	872	62	810	148
8	729	49	680	125	868	68	800	147
9	830	60	770	141	1094	64	1030	189
	816	51	765	140	1082	62	1020	187
10	777	77	700	128	1011	71	940	172
	886	61	825	151	932	62	870	159
11	802	62	740	136	958	73	885	162
	1036	56	980	180	841	71	770	141
Av.	759	68	691	127	831	75	756	139

[a] Barium borate-hydroxide incubation buffer.
[b] Sodium carbonate-bicarbonate incubation buffer.
[c] Klett-Summerson photoelectric colorimeter used, with 610 mμ filter.
[d] Differences of 6 scale divisions (0.5 γ) or less recorded as 0 γ phenol.

in artificial heat dehydration, and therefore a test for alkaline phosphatase activity was developed.

The test was found to be reliable and roughly quantitative with respect to the proportion of nondehydrated meal in a mixture. As little as 1% adulteration with nondehydrated material in high-quality, heat-dehydrated meal was detected regularly. The test therefore offers purchasers of alfalfa meal a valuable indication of nutritional quality.

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